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Anti-angiogenic effects of the water extract of HangAmDan (WEHAD), a Korean traditional medicine

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We investigated the anti-angiogenic effects of the water extract of HangAmDan (WEHAD), which is a crude extract of nine Korean medicinal substances of animal and plant origin. In human umbilical vein endothelial cells, WEHAD significantly inhibited bFGF-induced proliferation, adhesion, migration, and capillary tube formation. We used an antibody array to perform an analysis of signaling proteins, which showed up-regulated expression of various proteins including RAD51, RAD52, and p73, and down-regulated expression of pFAK. Blood vessel formation in a chick chorioallantoic membrane (CAM) treated with WEHAD was markedly reduced in length compared with a PBS-treated control group. These results suggest that inhibition of angiogenesis by WEHAD may be the mechanism of action for the anti-cancer effects of HAD.

angiogenesis, HangAmDan, WEHAD, antibody array, HUVEC

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Angiogenesis, which is the formation of new blood vessels from existing endothelium, is an essential process in various physiological and pathological situations including wound repair, organ regeneration, embryonic vascular system development and tumorigenesis. Tumor growth and metastasis are highly dependent on angiogenesis [1,2]. Treatments for human cancer that inhibit angiogenesis are the subject of intensive clinical investigation and, therefore, better understanding of the fundamental mechanisms of angiogenesis is critical in developing and improving these novel therapeutic options.

Angiogenesis is a complex process that involves the proliferation and migration of endothelial cells. It is delicately controlled by a variety of inducers and inhibitors that respectively cause the up- and down-regulation of angiogenesis [3,4]. It has been reported that most actively-studied basic fibroblast growth factors (bFGFs) and vascular endothelial growth factors (VEGFs) work as angiogenic inducers for both *in vivo* and *in vitro* growth [5–7]. Many kinds of angiogenic inhibitors have already been discovered including angiostatin, which decreases bFGF- and VEGF-mediated activation of MAPK in endothelial cells leading to inhibition of proliferation and induction of apoptosis [8]. Furthermore, Soamsan, a traditional Korean medicinal extract of herbal materials, has been shown to have anti-angiogenic

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effects and has been used to treat cardiovascular diseases [9]. Therapeutic inhibitors have been found using Proteo-Chip-based library screening methods. For instance, the A5-1 peptide that is effective as an angiogenesis inhibitor against integrin $\alpha5\beta1$ [10], and certain Korean medicinal plant extracts chosen under the same methodology have been verified to possess anti-angiogenic functions [11], have been identified using this methodology.

HangAmDan (HAD) is composed of nine products from species of Korean medicinal plants and animals (Table 1), and has been used for the treatment of cancer patients in oriental medicine. We have investigated how the water extract of HAD (WEHAD) inhibits bFGF-induced angiogenesis using assays to measure the proliferation, migration, and adhesion of human umbilical vein endothelial cells (HUVECs) *in vitro*, and an *ex vivo* chorioallantoic membrane (CAM) angiogenesis assay.

1 Materials and methods

1.1 Preparation of water extract of HAD

HAD was provided by the East-West Cancer Center of the Dunsan Oriental Medical Hospital of Daejeon University (Daejeon, Korea) (Table 1). The WEHAD was prepared by extracting HAD powder with 1 g of powder in 10 mL of distilled water at room temperature for 24 h. The extract was centrifuged at 1000×*g* for 30 min and then filtered and lyophilized. The extract powder was dissolved directly in distilled water.

1.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were provided from Innopharmascreen Inc. (Asan, Korea). Cells were cultured in a complete M199 medium (Invitrogen, CA, USA). The cells at passages 3–6 were used. HUVEC cultures were kept maintained routinely at 37°C in a humidified atmosphere of 5% CO_2, and used for assay at passages 3–6.

Table 1 Ingredients of HAD

Scientific name	Relative amount (mg)
Coix lachryma Semen	259.0
Panax notoginseng Radix	86.0
Hippocampus kelloggi	26.0
Cordyceps Militaris	26.0
Santsigu Tuber	26.0
Ginseng Radix	26.0
Bovis Calculus	17.0
Margarita	17.0
Moschus	17.0
Total amount (one capsule)	500.0

1.3 *In vitro* HUVEC proliferation assay

Assessment of cell proliferation was performed according to the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay protocol. HUVECs (1×10^4 cells/well) were added to 96-well tissue culture plates coated with gelatin and allowed to adhere overnight. The cells were treated with extract in the presence or absence of bFGF and incubated for 72 h. Then, 50 μL of a 1 mg mL^{-1} MTT solution was added to each well, and the cells were incubated for 2 h at 37°C. After the supernatants were discarded, residual formazan crystals were dissolved in 100 μL of DMSO. Absorbance was measured at 595 nm on an ELISA plate reader (Emax, Molecular Devices, USA). GRGDSP (PEPTRON, Daejeon, Korea), a synthetic peptide, is the peptide including Arg-Gly-Asp (RGD) in which the motif acts as various integrin-recognition motifs. It is extensively used as inhibitors of integrin-ligand interactions as well as in the study on cell adhesion, migration, growth and differentiation [12]. On the other hand, GRGESP (PEPTRON, Daejeon, Korea) is the peptide which loses the function of RGD by changing Asp of motif of RGD into Glu. It is also used as a control group along with RGD. The presented data were measured in triplicate.

1.4 *In vitro* HUVEC adhesion assay

An adhesion substrate was prepared by adding 100 μL of vitronectin (10 μg mL^{-1}; Sigma, St. Louis, USA) diluted in phosphate-buffered saline (PBS; pH 7.3) to 96-well ELISA plates (Nunc) overnight at 4°C. Each well was then washed twice with PBS to remove unbound vitronectin and blocked for 30 min at 37°C with 0.1% bovine serum albumin (BSA) in PBS. After blocking, the wells were washed three times with PBS.

HUVECs (5×10^5 cells mL^{-1}) were added to each well of a 96-well plate coated with fibronectin. HUVEC suspension (100 μL) including either fresh media (negative control) or WEHAD (30–1000 μg mL^{-1}) and bFGF (5 ng mL^{-1}) was added to three wells and incubated for 90 min at 37°C. After incubation, unbound cells were removed by rinsing each well twice with PBS and incubated for 1 h at 37°C with 100 μL 1% Coomassie blue solution (v/v) that diluted with MeOH. The optical density (*A*) at 595 nm of each well was measured using a spectrophotometer (Emax, Molecular Devices, USA). The presented data were measured in triplicate.

1.5 *In vitro* HUVEC migration assay

A migration assay was performed as previously [13,14]. HUVECs were placed in a modified Boyden chamber (48-well chemotaxis chamber AP48; Neuro Probe, Gaithersburg, MD, USA). Polycarbonate membranes (8 μm pore,

25 mm×80 mm in size, polyvinylpyrrolidine-free; Neuro Probe) were coated with gelatin and incubated overnight at 37°C. Basic FGF (5 ng mL^{-1}) in M199 was added to the lower chamber, and cell suspension containing 1×10^4 cells per 50 μL media and supplemented with the indicated concentration of medicinal extracts was added to the upper chamber of each well. The chamber was incubated for 8 h at 37°C with 5% CO_2 to allow cells to migrate through the gelatin-coated polycarbonate membrane. Non-migrating cells on the upper surface of the membrane were removed with a wiper tool (Neuro Probe), and the membrane was then stained with Diff-Quik (VWR Scientific Products, Bridgeport, NJ, USA). The total number per well of migrating cells with nuclei was determined as described. The evaluated data showed the relative cell migration, which is a percentage compared with the mean cell number for each stained field. The presented data were measured in triplicate.

1.6 *In vitro* capillary tube formation

Capillary tube formation of HUVECs was performed as described with some modification [15]. Twenty four-well culture plates were coated with 250 μL of Matrigel and allowed to solidify at 37°C for 30 min. HUVECs (2×10^5 cells) were added to each of three wells in 100 μL of media containing WEHAD and bFGF of 5 ng mL^{-1} (or fresh media as a negative control). After 18 h of incubation, the capillary tubes were fixed, stained with a Diff-Quick solution (Becton Dickinson, San Jose, CA, USA) and observed under a phase-contrast microscope.

1.7 *Ex vivo* bFGF-induced CAM angiogenesis

An *ex vivo* CAM assay was performed as previously described [16,17]. Fertilized eggs were incubated at 37°C in humidified air. Ovalbumin (4–5 mL) was extracted from the bottom of the eggs using a syringe on day 3, and windows were made within the tops of the eggs. The windows were sealed with transparent tape, and the eggs were further incubated until day 9. Thermonox (Nunc, Naperville, IL) containing bFGF (200 ng) or bFGF plus extract was implanted onto the CAM of an individual embryo. After a 48 h incubation period, intralipose (a white fat emulsion solution) was injected into the CAM for clear visualization of blood vessel formation, and each CAM was observed under a microscope and photographed.

1.8 Protein expression profiling in WEHAD-treated HUVEC

1.8.1 Preparation of cell lysates

When HUVECs reached confluence, they were serum-starved by incubation in M199 containing 1% FBS for 8 h. The cells were treated with or without WEHAD in the presence of growth factor. After 24 h incubation, the cells were washed twice with PBS and harvested in 5 mmol L^{-1} EDTA-PBS. The harvested cells were then maintained on ice for 10 min and centrifuged for 5 min at 1300 r min^{-1}. The pellets were washed with PBS and re-centrifuged.

1.8.2 Protein extraction and labeling

HUVECs were extracted with Lysis-M™ (Roche, Germany) mammalian cell extraction buffer. Each protein extract (100 μg, 1 mg mL^{-1}) was labeled with both Cy3 and Cy5 (GE Healthcare, UK) as per the manufacturer's instructions. Free dyes were removed with SigmaSpin columns (S5059, Sigma, USA) and purified samples were stored at 4°C until use.

1.8.3 Preparation of antibody microarrays

Forty-eight distinct antibodies against proteins involved in cell proliferation (Hypromatrix, Worcester, MA, USA) were spotted onto ProteoChip arrays in duplicate. The detailed procedure of array analysis has been previously described [18].

1.8.4 Hybridization

The fluorescence-labeled cell lysates were applied onto the antibody array and incubated for 1 h at 37°C in the dark. The slides were washed three times with PBST, N_2-dried and analyzed using a fluorescence microarray scanner.

1.8.5 Detection and data analysis

The antibody array slides were scanned using a GenePix 4100A microarray scanner (Axon Instruments, Union City, CA, USA) with 532 and 635 nm lasers. Image analysis was performed for each spot using the manufacturer's software package (GenePix 6.0, Axon Instruments). The INR (internally normalized ratio) of all spots were calculated as previously described [19].

1.8.6 Western-blot analysis

The cell pellets were lysed using Complete Lysis M (Roche). Protease and phosphatase inhibitor cocktails (both from Roche) were added. Protein extracts (50 μg) were separated on a 12% Bis-Tris Nupage gel (Invitrogen) and transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat milk, or BSA in PBS (pH 7.4) containing 0.5% Tween-20. It was then incubated with rabbit anti-FAK (1∶1000 dilution), rabbit anti-Rad51 (1∶1000 dilution), rabbit anti-p73 (1∶1000 dilution), or rabbit anti-GAPDH (1∶500 dilution) for 16 h at 4°C. The membrane was subsequently incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies for 60 min at room temperature. Protein bands were visualized on a medical X-ray film (Agfa) using an enhanced chemiluminescence (ECL™) kit (Invitrogen).

2 Results and discussion

2.1 Biological characterization of WEHAD effects on bFGF-induced HUVECs

To investigate the biological activities of WEHAD, we performed proliferation, migration, and adhesion assays using HUVECs. As an antagonistic extract, WEHAD markedly inhibited the proliferation of HUVECs compared with the PBS-treated control group (Figure 1A). A half-maximal inhibition of proliferation on the endothelial cells by WEHAD was observed at a concentration of 253 μg mL^{-1}.

To confirm the anti-angiogenic functions of WEHAD, an *in vitro* HUVEC adhesion assay and a migration assay were employed. WEHAD suppressed bFGF-induced cell attachment in a dose-dependent manner (Figure 1B). The anti-adhesive efficacy of WEHAD was higher than that of RGD. WEHAD also prevented bFGF-induced cell migration in a dose-dependent manner compared with the PBS control group (Figure 1C). To further assess the inhibitory effects of the antagonistic extracts on HUVEC proliferation, we carried out an *in vitro* capillary tube formation assay, and *ex vivo* CAM angiogenesis. Cells plated on Matrigel were treated for 16 h with WEHAD in the presence of bFGF and tube formation was observed using phase-contrast microscopy. WEHAD significantly suppressed tube formation at a concentration of 10 μg mL^{-1} (Figure 2A). The area of CAM below the disks without any treatment showed a normal density and branching pattern of the blood vessels, indicating that disk weight did not affect their growth. By contrast, the vascular branches of CAM treated with WEHAD (10 μg/egg) for 48 h were perturbed (Figure 2B). These findings are consistent with data obtained from HUVEC proliferation, adhesion, migration, and tubular network formation

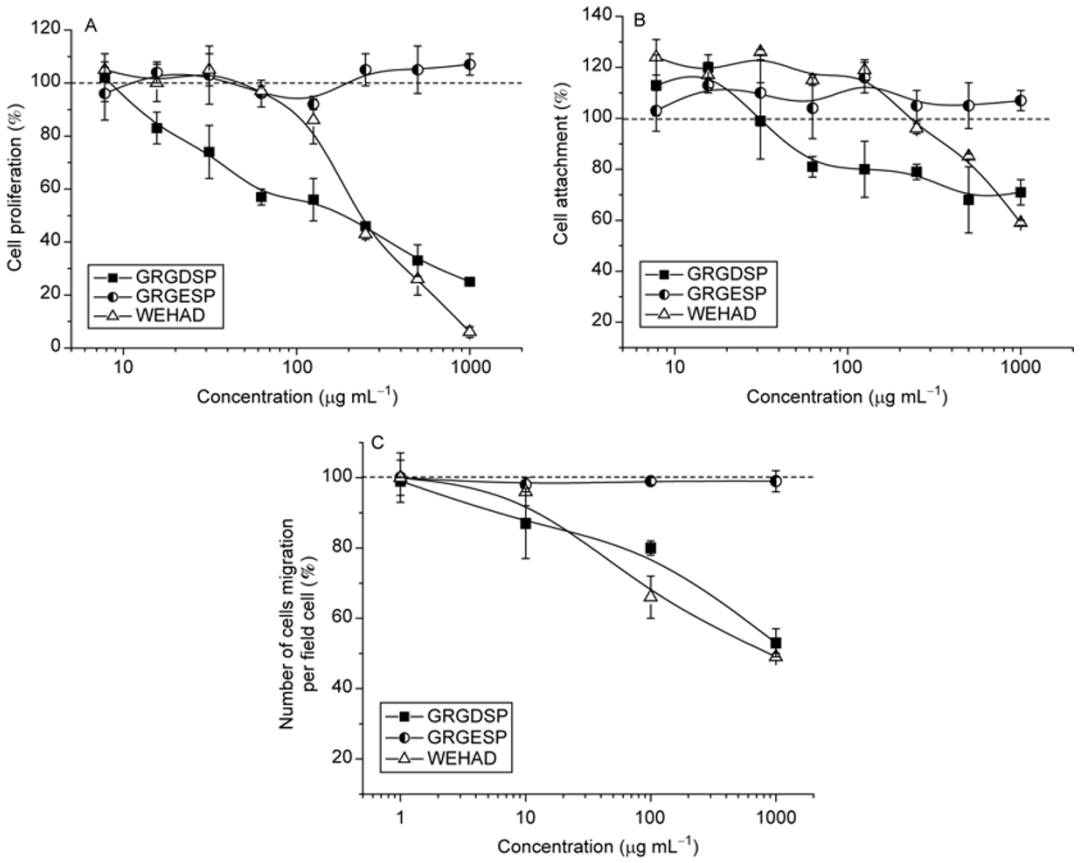
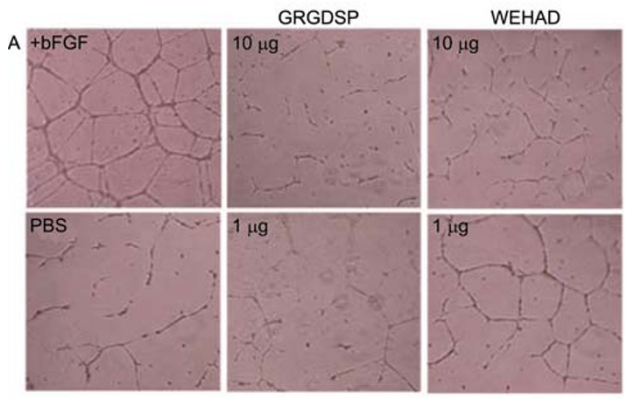
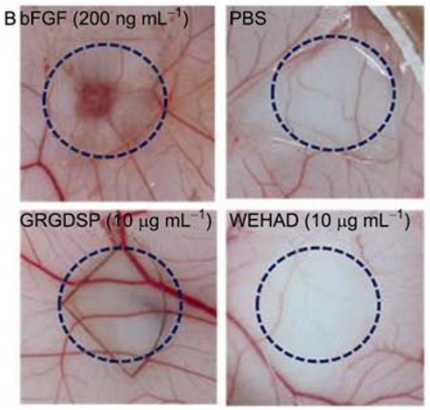


Figure 1 Inhibitory effect of WEHAD on HUVECs. A, Cell proliferation. HUVECs were incubated with different concentrations (7–1000 μg mL^{-1}) of WEHAD for 72 h in the presence of bFGF (5 ng mL^{-1}). After incubation, unbound cells were removed with PBS and incubated for 2 h with 100 μL of an MTT solution. Absorbance was measured at 595 nm using an ELISA reader. Control cells were incubated in the absence of bFGF and WEHAD, whereas bFGF-control cells were incubated in the presence of bFGF alone (dotted line). B, Attachment. HUVECs were incubated with different concentrations (30–1000 μg mL^{-1}) of WEHAD for 90 min in the presence of bFGF (5 ng mL^{-1}) on fibronectin-coated 96-well ELISA plates. After incubation, unbound cells were removed with PBS, and attached cells were fixed with methanol and stained with crystal violet. C, Migration. Dose-dependent inhibition of bFGF-induced HUVEC migration by WEHAD was examined. The total number of cells per field (*Y*-axis) was measured using an Image Gauge V2.54 program. Control cells were incubated in the absence of bFGF and WEHAD, whereas bFGF-control cells were incubated in the presence of bFGF alone (dotted line). GRGDSP and GRGESP were used as a positive and a negative control, respectively.





	Number of anti-angiogenic CAMs	CAM angiogenesis inhibition (%)
bFGF	0/10	0
PBS control	1/10	10
GRGDSP	9/10	90
WEHAD	9/10	90

Figure 2 Inhibitory effects of WEHAD on capillary tube formation and bFGF-induced CAM angiogenesis. A, HUVECs were seeded on Matrigel-coated 24-well plates in serum-free media containing GRGDSP or WEHAD in the presence of bFGF (5 ng mL^{-1}) or PBS alone. After 18 h, the capillary tubular network was stained with a Diff-Quick solution (Becton Dickinson, San Jose, CA, USA) and observed under a phase-contrast microscope. B, Thermonox discs containing bFGF alone, RGD (10 μg/egg), and WEHAD (10 μg/egg) in the presence of bFGF (200 ng/egg) were tested on each of 10 CAMs on nine-day-old chick embryos. After a 48 h incubation period, the CAMs were observed. A negative control was incubated in the absence of bFGF. GRGDSP was used as a positive control.

assays. Collectively, these results suggest that WEHAD could be useful for the development of a potent angiogenesis inhibitor.

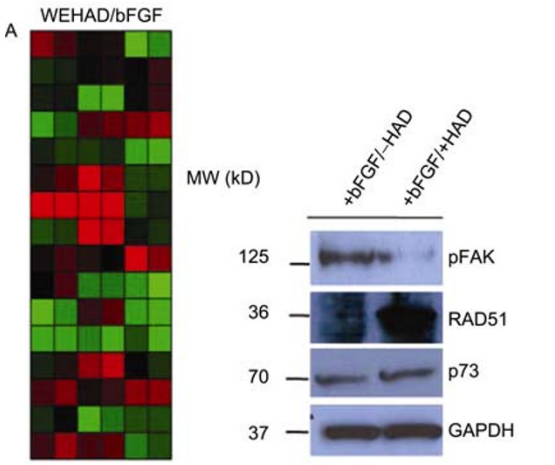
2.2 Expression profiling of cell cycle proteins in WEHAD-treated HUVECs using antibody microarray

The goal of this study was to analyze the expression pattern of endogenous signaling proteins in HUVECs treated with WEHAD. To investigate the protein expression profile in WEHAD-treated HUVECs, an antibody microarray constructed on ProteoChip was used. To carry out the antibody microarray analysis, WEHAD-treated HUVECs were labeled with Cy3 and Cy5.

Antibody microarray analysis of WEHAD-treated HUVECs labeled with Cy5 compared with the Cy3-labeled untreated HUVECs revealed up- or down-regulation of different proteins (Figure 3A and B). A further attempt was made to validate the antibody microarray data by immunoblot analysis. Selected up-regulated proteins such as RAD51, Cyclin B1, and p73 showed increased expression, whereas down-regulated proteins such as FAK showed a decrease in protein amount in Western blotting analyses (Figure 3C).

Focal adhesion kinase (FAK) is a widely expressed cytoplasmic protein tyrosine kinase involved in integrin-mediated signal transduction. It plays an important role in the control of several biological processes including cell spreading, migration, and survival [20]. Our antibody array data indicate that the inhibitory effect of WEHAD on the proliferation of HUVECs may be partly due to the decreased expression of FAK involved in integrin-mediated cell survival. p73, a member of the p53 family, is involved in cell cycle regulation, and induction of apoptosis. The p73-induced apoptosis is mediated by endoplasmic reticulum stress and induction of p53 up-regulated modulator of apoptosis (PUMA) [21,22]. Our finding suggests that the inhibition of HUVEC proliferation by WEHAD may be mediated by induction of apoptosis in the cells via increased p73 expression. It was reported that enhancement of p53 ubiquitination by the FAK FERM domain promotes cell proliferation and cell survival [23]. Based on our antibody array data, WEHAD treatment of HUVECs caused down-regulation of FAK as well as up-regulation of p73, resulting in suppression of cell proliferation. Additionally, the DNA repair protein, RAD51, was regulated by WEHAD in HUVECs. Collectively, these data suggest that WEHAD significantly inhibits bFGF-induced angiogenesis through the regulation of expression of certain proteins involved in cell growth.

In this study, we observed anti-angiogenic effects of WEHAD in HUVECs through different angiogenesis assays induced by bFGF. WEHAD had dose-dependent inhibitory effects on proliferation, migration and adhesion (Figure 1). We also verified this inhibitory effect through an *ex vivo* CAM assay (Figure 2B). Thus, WEHAD may have a potential therapeutic utility in the treatment of cancers involving neovascularization. However, as we already described, WEHAD is a natural product made up of nine types of plants and animals, i.e., it contains many types of compounds. Thus, further attempts will be made to examine the molecular mechanism correlated with cellular signaling pathways, and to identify the active compounds that appear to create the anti-angiogenic effects of WEHAD.



B

Raf-1	ERK2	pFAK
pERK1/2	MEK-1	MEK kinase-1
H-Ras	FAK	XRCC4
PIK	ERK1	IKB-b
Cyclin E	BRCA1	P16
P63	P73	C-MOS
RAD51	RAD52	CHK 1 kinase
GADD45 γ	DMC1	p53
Cip1/WAF-1/p21	PCNA	Cyclin B1
E2F-1	p35	Cdc6
Cdk2	Nibrin/Nbs1	Cullin-1
BRCA2	Cdc25A	Cyclin D
Cyclin A	Cdk4	Cdk1/cdc2
NFkBp52	Kip1 (p27)	STAT1
Kip2	STAT3	Rb2
pSTAT1	Sam 68	GAPDH

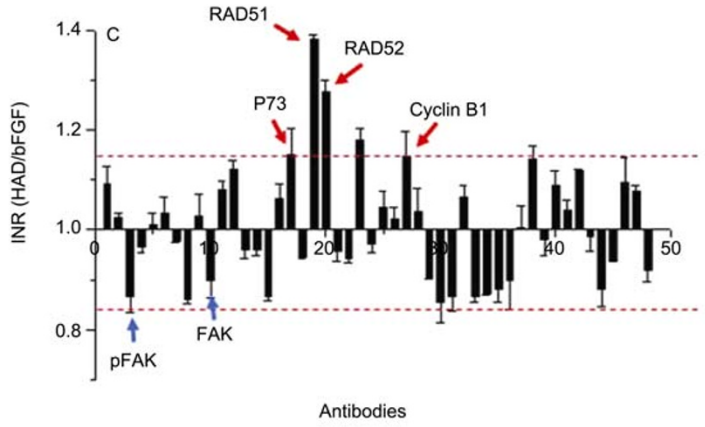


Figure 3 Analysis of differential expression of cell cycle proteins in HUVECs treated with WEHAD using the antibody microarray. A, Graded virtual image of Cy5:Cy3 ratios at spots within arrays of protein extracts from WEHAD-treated HUVECs and validation of antibody microarray data by immunoblot analysis. B, Map of the antibody chip. Up- and down-regulated proteins are shown by red and blue, respectively. C, Graphical representation of Cy5:Cy3 ratios on the antibody arrays. The bar chart shows the mean fluorescent intensity of three spots plotted over three independent experiments. Proteins having a normal median ratio in the range of 1.0 were considered as unchanged expression.

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